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13. ABSTRACT (Maximum 200 Words) Members of the Ras family of oncoproteins cause a high percentage of human cancers. Cancer proliferation can result from mutations in ras genes, overexpression of Ras proteins, or the aberrant activation of pathways that impinge on Ras signaling. Ras proteins must be posttranslationally modified with farnesyl and palmitoyl lipids to associate with the plasma membrane and exhibit transforming activity. Consequently, inhibitors of Ras farnesylation halt the growth of breast and other cancers. However, in addition to farnesylation, the palmitoylation of Ras cysteine residues by the enzyme palmitoyl acyltransferase (PAT) contributes to the transforming activity of Ras. Hence, the development of potent and selective inhibitors of PAT could define a novel class of anticancer agents. To identify compounds that affect Ras palmitoylation, we synthesized a novel fluorescent Ras-mimetic substrate. This and a related Src-mimetic substrate were used to investigate a small molecule inhibitor of PAT termed JF081204. To test the hypothesis that this inhibitor may exhibit anticancer activity by blocking palmitoylation of Ras proteins, we evaluated JF081204 as an anticancer agent, synthesized analogues to investigate structure activity relationships, and probed the mechanism of action of these compounds. We demonstrated that JF081204 inhibits PAT activity in platelets but does not inhibit palmitoylation of Ras in cancer cell lines.				
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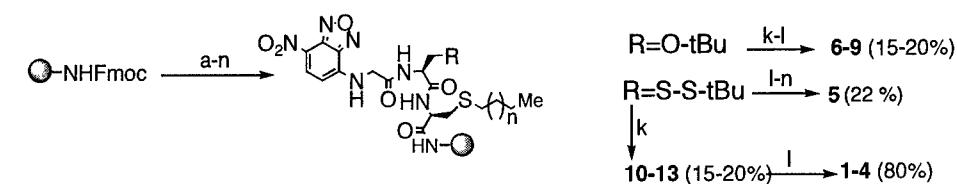
Introduction

Members of the Ras family of oncoproteins are responsible for the development of a high percentage of human cancers. The three human *ras* genes *H-ras*, *N-ras*, and *K-ras* encode small G proteins that relay signals from growth receptors at the cellular plasma membrane to the cell nucleus. The activation of Ras proteins is normally tightly controlled by regulatory proteins, but mutations in *ras* genes, overexpression of Ras proteins, or aberrant activation of pathways that impinge on Ras signaling can promote cellular transformation and result in the uncontrolled growth of cancer cells. Ras proteins must be posttranslationally modified with both hydrophobic farnesyl and palmitoyl lipids to associate with the cellular plasma membrane and exhibit transforming activity.¹ As a result, much effort in recent years has focused on the development of inhibitors of the enzyme farnesyl protein transferase (FPTase), which catalyzes the addition of the farnesyl lipid to Ras proteins.² Inhibitors of FPTase are currently in clinical trials that block this lipid modification and halt the growth of a variety of cancers including breast cancers.³ However, in addition to farnesylation, the palmitoylation of cysteine residues of Ras proteins by the enzyme palmitoyl acyltransferase (PAT) is also critical for the transforming activity of Ras. Hence, the development of potent and selective inhibitors of PAT could define a novel class of anticancer agents. To investigate and identify these compounds, we developed a new assay of Ras palmitoylation by synthesizing a fluorescent mimic of Ras proteins. We also synthesized the novel PAT inhibitor JF081204 and evaluated this and related compounds as anticancer agents and inhibitors of palmitoylation in platelets.

Body

Synthesis of Ras-mimetic substrates of palmitoyl acyltransferase (PAT).

Our laboratory previously reported a fluorescent substrate of PAT that mimics the N-terminus of Src-family oncoproteins.⁴ We hypothesized that related fluorescent substrates that mimic the C-terminus of Ras proteins might provide additional useful tools for studies of inhibitors of protein palmitoylation. To test this hypothesis, we synthesized on solid phase compounds that mimic the C-terminus of the yeast Ras2 protein. These compounds comprised the general structure NBD-Gly-Cys-Cys(alkyl)-CONH₂. The NBD group provided a green fluorophore, and the alkyl groups corresponded to butyl, octyl, decyl, and hexadecyl alkanes. The length of these appended alkyl groups proved to be critical for the activity of these compounds. The substrate bearing the octyl side chain exhibited the greatest activity in whole cell (Jurkat lymphocyte) assays of palmitoylation (Figure 2). The other compounds were not efficiently palmitoylated in these assays. To confirm that NBD-Gly-Cys-Cys(octyl)-CONH₂ was palmitoylated in whole cells, cellular extracts of Jurkat lymphocytes treated with this substrate and ¹⁴C-palmitic acid and analyzed by thin layer chromatography and autoradiography (Figure 3). The Ras-mimetic NBD-Gly-Cys-Cys(octyl)-CONH₂ compound provided a novel whole-cell assay of inhibitors of protein palmitoylation.



(a) Piperidine, DMF (b) Fmoc-Cys(S-S-tBu)-OH, PyBOP, HOBt, DIEA (c) HOCH₂CH₂SH, DIEA (d) 1-Iodoalkane, DIEA (e) Piperidine, DMF (f) Fmoc-Cys(S-S-tBu)-OH or Fmoc-Ser(tBu)-OH, PyBOP, HOBt, DIEA (g) Piperidine, DMF (h) Fmoc-Gly-OH, PyBOP, HOBt, DIEA (i) Piperidine, DMF (j) NBD-Cl, Et₃N, DMF (k) TFA (l) HOCH₂CH₂SH, DIEA (m) palmitoyl-Cl, DIEA (n) TFA

Figure 1. Synthesis of a novel Ras-mimetic substrate of protein palmitoylation.

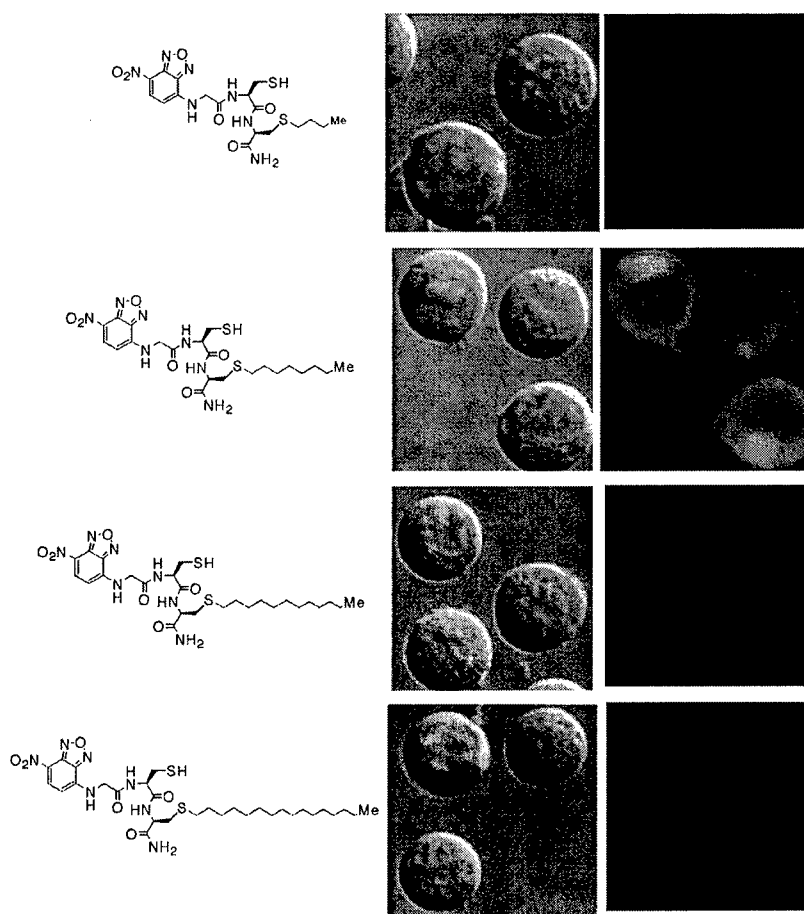


Figure 2. Differential interference contrast (left panels) and epifluorescence microscopy (right panels) of Ras-mimetic compounds added to living Jurkat lymphocytes for 2 h at 37°C. Only the S-Cys-octyl compound (second from the top) associated with plasma membranes of treated cells.

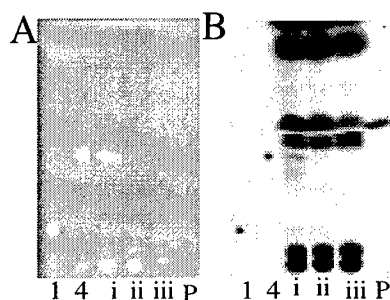
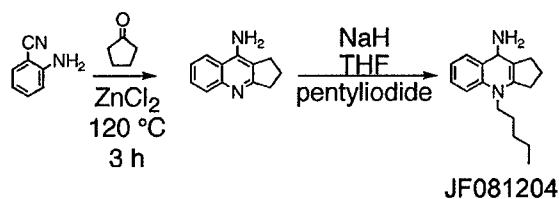


Figure 3. Autoradiography of cellular extracts to verify palmitoylation of the Cys-octyl Ras-mimetic probe. TLC of extracts of Jurkat lymphocytes treated with **2** and ^{14}C -palmitic acid. (A) Fluorescent products imaged by UV-irradiation (365 nm). (B) Autoradiography of the TLC plate. i: Extract of cells treated with **1** (1 μM). ii: Extract of cells treated with **2** (1 μM) and 2-bromopalmitic acid (100 μM). iii: Extract of cells treated with ^{14}C -palmitic acid alone. P: ^{14}C -palmitic acid. After elution, **2** and **5** in control lanes were marked with ^{14}C -palmitic acid to enable detection on film.

Synthesis of JF081204 and analogues.

Compound JF081204 was discovered by Prof. Robert Flaumenhaft at Harvard Medical School as an inhibitor of platelet activation ($\text{IC}_{50} = 5 \mu\text{M}$). This compound was identified by screening the commercially available ChemBridge combinatorial library of drug-like molecules. JF081204 was hypothesized to function by mimicking the PAT cofactor palmitoyl-CoA. As part of a collaboration with the Flaumenhaft laboratory, we successfully synthesized JF081204 and analogues using the approach shown in Figure 4. These compounds were investigated as inhibitors of Ras palmitoylation in cancer cell lines and platelets.



Related analogues of JF081204 :

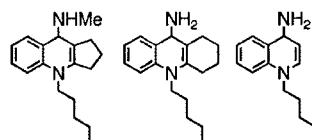


Figure 4. Synthesis of JF081204 and related analogues (other analogue structures are also shown in Figure 5).

Evaluation of JF081204 and analogues as inhibitors of protein palmitoylation in cancer cell lines.

JF081204 and analogues were evaluated as inhibitors of protein palmitoylation using our previously reported Src-mimetic substrate⁴ and our new Ras-mimetic substrate in Jurkat lymphocytes, MCF7 breast cancer cells, and MDA-MB-468 breast cancer cells. However, none of these compounds were active in these assays at concentrations of up to 100 μ M (Figure 5). The effects of these compounds on the proliferation of MCF7 and MDA-MB-468 breast cancer cell lines were also evaluated using trypan blue exclusion assays, but none of these compounds exhibited cytotoxic or antiproliferative effects against these breast cancer cell lines.

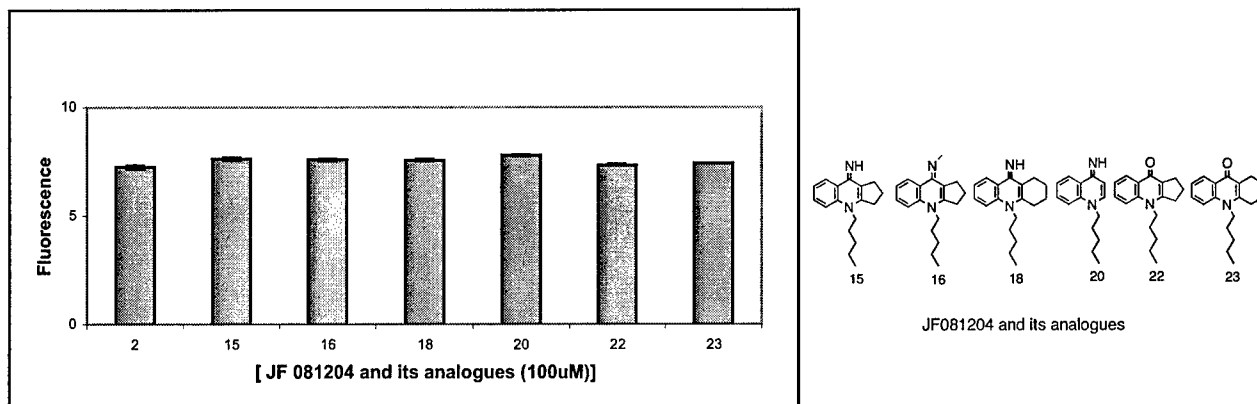


Figure 5. Representative negative results showing lack of inhibition of protein palmitoylation in MCF7 breast cancer cells treated with DMSO alone (compound 2), JF081204 (compound 15) and analogues shown to the right (100 μ M). Palmitoylation was assayed by flow cytometry using cells treated with the Cys-octyl Ras-mimetic substrate (10 μ M).

Evaluation of JF081204 as an inhibitor of protein palmitoylation in platelets.

Synthetic JF081204 provided to the laboratory of Prof. Robert Flaumenhaft was shown to inhibit the incorporation of ³H palmitate into platelet proteins (Figure 6). This compound also blocked palmitoylation of our Src-mimetic substrate in platelets (Figure 7). The JF081204 compound appears to be a specific inhibitor of a palmitoyl acyltransferase expressed in platelets.

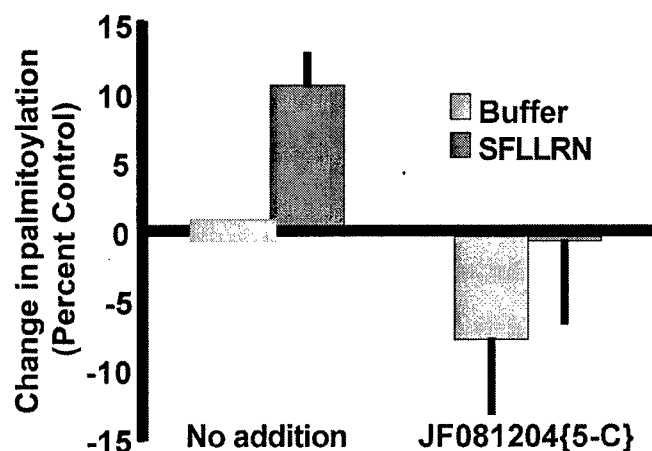


Figure 6. JF081204 {5-C} inhibits incorporation of [3 H]-palmitate into platelet proteins. Platelets were labeled with [3 H]-palmitate in the presence or absence of 20 μ M JF081204{5C} as indicated. Platelets were subsequently incubated in the presence or absence of the SFLLRN peptide. Platelet proteins were precipitated using ice-cold acetone and extracted exhaustively using chloroform:methanol:water. Covalently linked [3 H]-palmitate was quantified by scintillation counting.

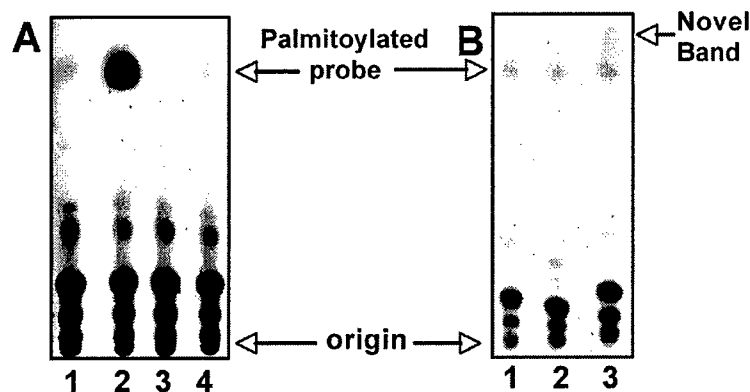


Figure 7 Effect of incubation of JF081204 compounds on palmitoylation of the fluorescent palmitoylation probe. A) Platelets exposed to 10 μ M palmitoylation probe were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 5 μ M JF081204{5-C} for 15 minutes. Samples were then incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 μ M SFLLRN. Extracts of samples were subsequently analyzed by TLC and the probe was visualized using a molecular imager. B) Platelets exposed to 10 μ M palmitoylation probe were incubated with either DMSO vehicle (lane 1), 100 μ M JF081204{5-C}, or 30 μ M JF081204{12-C} for 15 minutes. Extracts of samples were subsequently analyzed by TLC and the probe was visualized using a molecular imager. A novel band was observed upon incubating platelets with JF081204{12-C}. The origin and migration of the palmitoylated probe as analyzed by TLC are indicated.

Key Research Accomplishments

Key research accomplishments include (1) the synthesis of a novel fluorescent Ras-mimetic substrate of protein palmitoylation, (2) the synthesis of JF081204 and analogues, and (3) the determination that JF081204 is an inhibitor of protein palmitoylation in platelets.

Reportable Outcomes

Data obtained from this research was included in a grant submitted to the National Institutes of Health entitled "Thrombosis and Platelet Disorders" (Bruce Furie, Principal Investigator, Beth Israel Deaconess Medical Center and Harvard Medical School; Project #2: Novel Inhibitors of Platelet Function, Robert Flaumenhaft, Project Leader, Blake R. Peterson, Collaborator).

Conclusions

Assays of protein palmitoylation were developed from a fluorescent Ras-mimetic substrate of palmitoyl acyltransferase. The small molecule JF081204 was found to inhibit palmitoyl acyltransferase activity in platelets but not in cancer cell lines.

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Appendices - None